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Cheeky Business: Comparing DNA Yield of Buccal, Fecal, and Whisker Samples for Minimally Invasive Genetic Research

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Abstract

Conservation research concerning rare species can be arduous. At the same time, assessing the genetic characteristics of a population is critical for informing conservation and management strategies. For species that are rare or threatened, researchers must balance the well-being of the animal and its population size with the need to acquire adequate samples for genetic analyses. Ord's kangaroo rats (*Dipodomys ordii*) were live-trapped to compare DNA yield between three minimally invasive sampling techniques: whisker extraction, buccal swabbing, and fecal pellet collection. Although fecal pellets yielded higher DNA concentrations than either buccal swabs or whisker follicle extraction, additional laboratory steps may be required for successful PCR. This suggests that whisker samples might be a more desirable means of collecting and isolating DNA from animals for minimally invasive studies. Given these promising results, it appears that minimally invasive means of gathering DNA, such as whisker collection, should continue to be refined and employed in genetic research of rare and threatened mammals.

Key words: conservation genetics, *Dipodomys ordii*, small mammals

Introduction

Guidelines for determining the conservation status of rare and endangered species include evaluation of several population biology parameters (Soule 1985). For example, assessing population genetic structure can provide insights into taxonomic status, genetic health, and presence/absence of cryptic species (Avise 1989). For rare taxa, however, evaluating intraspecific population substructure can be difficult due to issues inherent with small population sizes. First, sampling logistics may be challenging because these species are often rare and difficult to capture. Second, the challenge of collecting sufficient, high-quality DNA from rare species must be balanced with minimizing harm to the animals (Waits and Paetkau 2005).

Herein, DNA yield from buccal swabs, whisker extractions, and fecal pellet collection, three of the least invasive DNA collection methods for mammals (Meldgaard et al. 2004), was quantified to identify the most efficient means of non-invasively obtaining genetic material during field sampling. DNA isolated from liver samples from voucher specimens were used

as an internal control for assessing DNA quality and quantity. *Dipodomys ordii* is a kangaroo rat with a relatively large distribution that extends from southern Canada into Mexico, and from eastern California and Oregon to central Oklahoma and Kansas (Garrison and Best 1990). *Dipodomys ordii* was selected as the representative taxon for two reasons: 1) high relative abundance throughout most of its geographic distribution facilitates collection and analysis of tissue samples; and 2) results from this species can be applied to other rodents, and particularly to other species within *Dipodomys*, a genus where nearly one-third of its members currently hold IUCN designations above "least concern" (IUCN Red List 2020).

In this assessment of three minimally invasive collection methods, whiskers were chosen over fur or tail hair for two reasons: 1) whisker follicles tend to be larger than fur or tail hair follicles; and 2) though hair snares have been developed for other small mammals such as the American pika, *Ochotona princeps* (Henry and Russello 2011), hair snares typically are used for minimally invasive DNA collection in elusive medium to large sized mammals.

METHODS

To examine which of the minimally invasive methods yielded the most high-quality DNA per sample, *D. ordii* individuals were collected at multiple locations in north-central Texas in 2016. Guidelines established by the American Society of Mammalogists (Sikes et al. 2016) were followed, and the protocols used within were approved by the Institutional Animal Care and Use Committee at Texas Tech University (#T14083).

Appropriately "scruffing" each animal by a trained and experienced handler, two whiskers were extracted, one from either side of the rostrum, using sanitized forceps. Care was taken to select thicker whiskers (i.e., macrovibrissae) and to include the follicle by grasping the whisker close to the rostrum. Whiskers were stored in 1% sodium dodecyl sulfate (SDS) lysis solution (Longmire et al. 1997). As with harvesting of whiskers, two individuals participated in the collection of buccal swabs. One person "scruffed" the animal and the other administered the swab (Puritan Medical Products Company, LLC; Guilford, ME). Buccal swabs were collected by swabbing one side of the interior of the cheek, and not the cheek pouch, for 10 seconds with a sterile cotton swab. The swab shaft was broken about 2 cm from the head of the swab and placed head down into 1% SDS lysis solution. Fecal samples were collected in situ from Sherman traps. After an individual was determined to be our target species, the animal was removed from the trap, and fresh fecal pellets were dumped onto a sterile surface. With forceps disinfected with a 75% ethanol wash, we collected 10 fecal samples per individual and stored these in a vial containing 75% ethanol. All samples were flash-frozen in liquid nitrogen. Upon returning to the lab (typically 24–96 h later), samples were transferred to a -80°C freezer. Liver samples were collected from 10 *D. ordii* specimens, and later deposited in the Natural Science Research Laboratory at the Museum of Texas Tech University. Identification numbers of all specimens can be found in Table 1.

One measure of efficiency is the ease at which DNA can be extracted from a variety of materials using basic extraction kits. DNA extraction for all sample types was accomplished with the Qiagen DNeasy Blood and Tissue spin column protocol (Qiagen, Venlo, Netherlands) using the manufacturer's instructions but with the following adjustments. For each whisker, the follicle was cut away from the whisker shaft using scissors cleaned with 50% bleach and 70% ethanol and placed in a new microcentrifuge tube. One hundred and eighty microliters of the sample's SDS lysis solution and 20 µL of proteinase K were added to each tube. When transferring to the spin column, any remaining follicle was removed to avoid clogging the column. For buccal swabs, the swab was removed from the vial, and approximately 20 µL of lysis solution was pipetted directly from the swab and placed in a microcentrifuge tube. As with the whiskers, 180 µL of the sample's lysis solution was added along with 20 µL of proteinase K. Three fecal pellets per individual, after rinsing off excess ethanol with MilliQ water, were placed in a microcentrifuge tube with 180 µL of Qiagen tissue lysis buffer and 20 µL of proteinase K. For the reference samples, no more than 0.5g of liver tissue was placed in

Table 1. All samples used in the analysis, including individuals from field efforts and those collected as voucher specimens. Voucher specimens were deposited in the Natural Science Research Laboratory at the Museum of Texas Tech University in Lubbock, Texas.

Sample ID	Collection Type	Locality (county provided, if voucher specimen)	Tissue	Average DNA concentration in ng/µl	Average 260/280 nm absorbance values
TX-RODX 1001	field	north-central Texas	whisker	0.243	negative
TX-RODX 1005	field	north-central Texas	whisker	0.265	1.85
TX-RODX 1007	field	north-central Texas	whisker	0.154	1.77
TX-RODX 1009	field	north-central Texas	whisker	0.387	abnormal (97.04)
TX-RODX 1015	field	north-central Texas	whisker	1.580	1.88
TX-RODX 1017	field	north-central Texas	whisker	0.716	1.91
TX-RODX 1019	field	north-central Texas	whisker	0.425	1.96
TX-RODX 1021	field	north-central Texas	whisker	0.338	1.16
TX-RODX 1023	field	north-central Texas	whisker	0.266	1.78
TX-RODX 1025	field	north-central Texas	whisker	0.313	1.77
TX-RODX 1027	field	north-central Texas	whisker	0.707	1.52
TX-RODX 1029	field	north-central Texas	whisker	0.459	1.4
TX-RODX 1031	field	north-central Texas	whisker	0.169	1.65
TX-RODX 1035	field	north-central Texas	whisker	0.061	1.3
TX-RODX 1002	field	north-central Texas	buccal	0.033	0.31
TX-RODX 1004	field	north-central Texas	buccal	0.143	0.46
TX-RODX 1006	field	north-central Texas	buccal	0.093	1.9
TX-RODX 1010	field	north-central Texas	buccal	0.051	0.06
TX-RODX 1012	field	north-central Texas	buccal	0.179	2.06
TX-RODX 1016	field	north-central Texas	buccal	0.737	1.24
TX-RODX 1018	field	north-central Texas	buccal	0.561	negative
TX-RODX 1020	field	north-central Texas	buccal	0.044	n/a
TX-RODX 1022	field	north-central Texas	buccal	0.057	2.00
TX-RODX 1024	field	north-central Texas	buccal	0.082	negative
TX-RODX 1026	field	north-central Texas	buccal	0.249	n/a
TX-RODX 1028	field	north-central Texas	buccal	0.072	0.51
TX-RODX 1030	field	north-central Texas	buccal	0.063	4.48
TX-RODX 1032	field	north-central Texas	buccal	0.142	n/a
TX-RODX 1034	field	north-central Texas	buccal	0.112	2.38

Table 1. (cont.)

Sample ID	Collection Type	Locality (county provided, if voucher specimen)	Tissue	Average DNA concentration in ng/µl	Average 260/280 nm absorbance values
TX-RODX 1036	field	north-central Texas	buccal	0.022	0.76
TX-RODX 1039	field	north-central Texas	buccal	0.177	negative
TX-RODX 1058	field	north-central Texas	buccal	0.130	negative
6.1	field	north-central Texas	fecal	13.30	1.45
11.2	field	north-central Texas	fecal	12.80	1.56
13.1	field	north-central Texas	fecal	3.39	1.26
14.3	field	north-central Texas	fecal	4.80	1.67
16.1	field	north-central Texas	fecal	15.60	1.75
17.1	field	north-central Texas	fecal	9.54	1.36
18.1	field	north-central Texas	fecal	10.50	1.04
18.2	field	north-central Texas	fecal	11.17	1.37
18.4	field	north-central Texas	fecal	8.84	1.62
19.1	field	north-central Texas	fecal	below assay range	1.35
22	field	north-central Texas	fecal	8.56	1.78
TK249581	voucher	Motley, TX	liver	57.63	1.72
TK249582	voucher	Childress, TX	liver	51.77	1.77
TK249583	voucher	Childress, TX	liver	193.00	1.85
TK249584	voucher	Hall, TX	liver	234.00	1.86
TK249585	voucher	Hall, TX	liver	387.67	1.97
TK249586	voucher	Hall, TX	liver	148.00	1.9
TK249587	voucher	Motley, TX	liver	155.33	1.81
TK249590	voucher	Childress, TX	liver	390.33	1.74
TK249591	voucher	Hall, TX	liver	75.90	1.85
TK249592	voucher	Childress, TX	liver	258.33	1.81

a microcentrifuge tube with Qiagen tissue lysis buffer and proteinase K. In all cases, we incubated samples at 56°C for at least 8 hours prior to extraction. Also, the final elution volume was decreased from 200 µL to 100 µL, in all but liver samples, in attempts to recover as much concentrated DNA as possible. Two 100 µL elutions were performed for each sample. DNA extraction for tissue samples followed the standard Qiagen DNeasy protocol.

All samples were quantified using the dsDNA setting, high sensitivity (HS) assay on a Qubit 3.0 fluorometer (Life Technologies; Carlsbad, CA). Additionally, purity of samples was evaluated using a NanoDrop

1000 spectrophotometer (Thermo Scientific, Wilmington, DE) which returns 260/280 nm absorbance ratios. These ratios indicate purity of DNA, with values of approximately 1.8 considered pure. Each quantification method was performed in triplicate.

To examine whether the DNA collected was sufficient for downstream genetic analyses, a portion of the cytochrome-*b* gene was amplified using standard polymerase chain reaction with a thermal profile of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds for 32 cycles with a final elongation step at 72°C for 5 minutes. Reagent volumes for a 25 µL reaction were as follows: 15 µL water, 0.5 µL dNTPs, 0.5 µL forward primer, 0.5 µL reverse primer, 2µL $MgCl_2$, 2.5 µL 10X Taq buffer, 1.2 µL Taq polymerase, and 2.5 µL DNA template. The forward primer was Wd-400 5'-CCA TGA GGA CAA TAT CCT TCT GAG GG-3' (Edwards et al. 2001), and the reverse primer was in the control region for *Dipodomys ordii*, 5'-GTA CGT GTC ACG GAA AAT CT-3' (Thomas et al. 1990). The target amplicon size was approximately 1,100 base pairs.

A one-way ANOVA was conducted to compare DNA concentrations among buccal swabs (n = 18), whisker extractions (n = 14), liver samples (n = 10), and fecal pellets (n = 11). Additionally, a Kruskal-Wallis rank sum test was conducted between buccal swabs (n = 12), whisker extractions (n = 12), fecal pellets (n = 12), and liver samples (n = 10) to compare 260/280 nm absorbance ratios. Negative or abnormal 260/280 nm absorbance ratios (for example, one sample had a reading > 90), are not uncommon when quantifying small quantities of DNA (< 10 ng/µL; Thermo Scientific 2012). These were excluded from the 260/280 nm absorbance analysis. All statistical analyses were performed in R (R Core Team 2019).

RESULTS

Average DNA concentrations and 260/280 nm absorbance ratios are provided in Table 1. Assuming equal variances (Levene's test; $F_{3,49} = 0.081$, $P = 0.50$), DNA concentrations in ng/µL varied across tissue types ($F_{3,49} = 222.3$, $P = < 0.0001$). The post-hoc analysis revealed significant differences across all pairwise comparisons (Fig. 1a). Results of the Kruskal-Wallis test indicated that 260/280 nm absorbance ratios between all groups were not statistically significantly different ($\chi 2 = 4.66$, $df = 3$, $P = 0.199$). Compared to whisker and liver sample types, 260/280 nm absorbance ratios of buccal swab samples showed greater variability (Fig. 1b).

DISCUSSION

As would be expected from extractions using less initial material, minimally invasive DNA extractions from buccal swabs and whisker extractions yielded substantially lower DNA quantities than tissues, such as liver (Fig. 1a). However, DNA extracted from buccal swabs and whisker extractions provide sufficient material for downstream applications appropriate for genetic research on rare and threatened species (Fig. 1c). Additionally, these results suggest that minimally invasive sampling protocols potentially could be applied to conservation genomics techniques, such as restriction site associated DNA sequencing or other reduced representation techniques (unpublished data), whole genome sequencing, DNA archival, or studies of present and past demographics (McMichael et al. 2009; Avise 2010; Koboldt et al. 2013; McMahon et al. 2014).

In addition to increased DNA yield from whiskers compared to buccal swabs, other considerations become apparent. It has been documented in C3H mice (*Mus musculus*) and Wistar rats (*Rattus norvegicus domestica*) that whiskers grow back about 8–11 days after extraction (Ibrahim and Wright 1975), therefore causing little lasting harm to the individual. Second, whisker collection from a live rodent takes less effort than swabbing the inside of a rodent's cheek. It is likely less stressful on the animal and may also reduce the risk of bites to the handler. Stored food or mishandling of

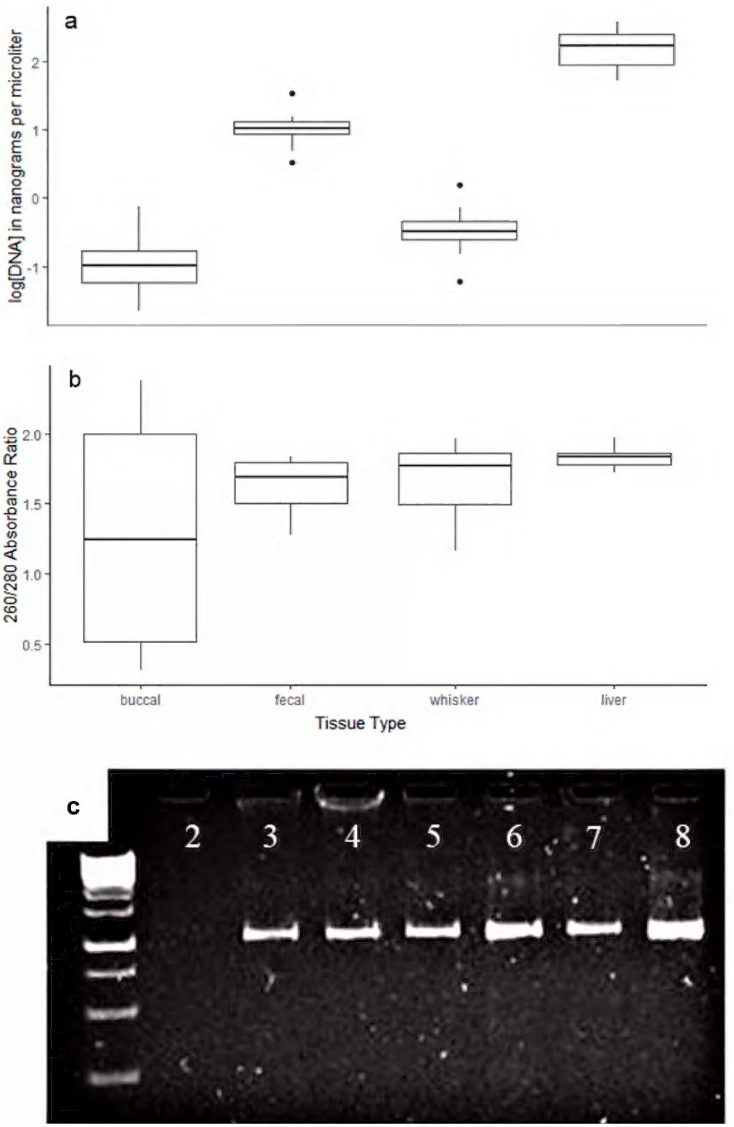

Figure 1. Quantification and gel electrophoresis of buccal, fecal, whisker, and liver samples. a) DNA quantity in ng/µl of buccal (n = 18), fecal (n = 11), whisker (n = 14), and liver (n = 10) samples from *Dipodomys ordii*. Data were log transformed prior to statistical analysis. b) Boxplot showing 260/280 absorbance ratios for samples that provided non-negative readings and non-abnormal readings: buccal (n = 12), fecal (n = 12), whisker (n = 12), and liver (n = 10). c) Results of PCR amplification of the cytochrome-*b* gene. Lanes are as follows: 100 base pair ladder (unlabeled), water (2), liver (3), four lanes of whisker samples (4-7: samples TXRODX 1015, TXRODX 1017, TXRODX 1019, TXRODX 1027), and a buccal swab sample (8: sample TXRODX 1012). Fecal pellet amplifications were unsuccessful.

the swab on part of the researcher (e.g., by swabbing the incisors) could introduce sources of contamination when conducting buccal swabs. This might lead to the increase of sample quality variability. Furthermore, in desert-adapted species such as kangaroo rats, saliva may be less available, yielding fewer cells for DNA extraction.

For some animals that are critically imperiled, in many situations it is not permitted to capture or handle the animal, which would be necessary for whisker extraction and buccal swabbing. Therefore, DNA collection from fecal material might be the only alternative. Techniques for extracting DNA from fecal material are readily available in the literature (Zhang et al. 2006). Many methods were tested on larger mammals, whose fecal deposits tend to be conspicuous in the field, such as those in mammalian families Cervidae, Canidae (Ramón-Laca et al. 2015), or Ursidae (Bellemain et al. 2005). Consequently, it was evaluated whether DNA could be amplified from opportunistically collected kangaroo rat fecal pellets under standard laboratory conditions. Initially, these fecal pellets were gathered for another investigation and therefore were not treated optimally for DNA extraction. As such, these amplifications were unsuccessful, even after modifying our PCR protocol. DNA was re-isolated from fecal pellets using one pellet instead of three to minimize the concentration of potential PCR inhibitors in the sample. Fecal samples are notorious for containing PCR inhibitors (Waits and Paetkau 2005) despite nearly pure 260/280 nm absorbance ratios. Solutions to counteract PCR inhibitors in feces include use of a specialized fecal kit (e.g., QIAamp PowerFecal DNA Kit), additional PCR cycles, or additions of reagents (e.g., adding bovine serum albumin or BSA). Fecal kits are not only more expensive but also more time-consuming (Fernando et al. 2003). Moreover, DNA from more than just the target species will be included in the sample (Bradley and Vigilant 2002), though primers designed to amplify the target sequence can alleviate this. Barring contamination, DNA isolated from whisker extractions should contain material only from the individual of interest. Increasing the number of PCR cycles, as may be necessary for amplifying DNA from fecal samples, introduces the added risk of spurious amplification of any other DNA in the sample. Though fecal amplification in the present study was unsuccessful, other researchers have had success (Galan et al. 2012; Verkuil et al. 2018). However, if fecal samples were collected in a manner that does not facilitate DNA amplification, and if permits allow handling of the animals, isolating amplifiable DNA from whisker follicles under typical laboratory conditions is a less troublesome process than amplification from opportunistically collected fecal pellets.

Genetic sampling from threatened populations raises unique concerns. Taking blood, toe clips, or voucher specimens and associated tissues is often not ideal because of the stress that it can cause to the animals and potentially to the size of the population. With much biological information to gain from genetic analysis, such as estimates of effective population size or detection of population substructure, it is imperative that minimally invasive procedures, such as those discussed in this paper, continue to be developed and implemented.

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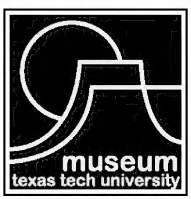

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